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⑯ Peptide, process for synthesizing it, pharmaceutical composition containing it and use.

⑯ A peptide having the following structure:  
His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-  
Arg-Lys-Gln-X-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-  
Gly-Y  
(wherein X is Met or Leu; and Y is OH, Lys-OH, Arg-OH, Lys-  
Arg-OH, or Lys-NH<sub>2</sub> when X is Met, or Y is Lys-NH<sub>2</sub>, Lys-OH, or  
Lys-Arg-OH when X is Leu), a process for synthesizing it, a  
pharmaceutical composition containing it, and its use in the  
preparation of a medicament having bronchodilative and hy-  
potensive activity are disclosed.

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PEPTIDE, PROCESS FOR SYNTHESIZING IT,  
PHARMACEUTICAL COMPOSITION CONTAINING IT AND USE

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The present invention relates to a novel physiologically active peptide having bronchodilative and hypotensive activities. Thus, the present invention is utilized in a field of drugs for medical treatment.

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VIP (vasoactive intestinal polypeptide), which was isolated by S. Said et al., has an amino acid sequence analogous to those of secretin, glucagon and the like, and hence is classified as a peptide belonging to the glucagon family. Its physiological activities cover such a wide range that they are observed in many fields of, for example, the

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cardiovascular system, the respiratory system, the metabolic system, and the endocrine system. Recently, the function of VIP as a neurotransmitter has also attracted attention. The function of VIP in the respiratory system should be particularly noted.

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Specifically, VIP has a very strong function of relaxing the smooth muscles of a bronchus so that it can very well relax smooth muscles contracted by a stimulative substance such as acetylcoline, histamine, or serotonin. This relaxation function is characteristically different from those of ordinary bronchodilators, which act through the  $\beta_2$ -receptor

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of adrenaline, in that it is not inhibited by the  $\beta$ -blocker. This well suggests a possibility that VIP and its derivatives may exhibit a tremarkable effect on a fit of intractable asthma on which a  $\beta_2$ -receptor stimulant does not effectively act.

5 The following articles will be mentioned as documents describing the above-mentioned findings.

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(a) S. Said, V. Mutt, Nature 225, 863 (1970).

(b) N. Hara, A. Guemei, S. Said et al., Clin. Res. 23, 347A (1975).

15 (c) Kudo, Sogo Rinsho 34 (11), 2497 (1985).

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VIP is a peptide having an amidated C-terminal and consisting of 28 amino acids as represented by the primary structural formula which will be mentioned below. Since the content of VIP in vivo is low, a large amount of VIP cannot be obtained by extraction of a natural matter. The synthesis of the peptide by the liquid-phase or solid-phase process is so expensive that it may be difficult to put it into practice. Since the C-terminal is amidated, production of VIP by gene recombination is technically difficult and expensive.

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His-Ser-Asp-Ala-Val-Phe-Thr-Asp-

Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-

Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-

Ser-Ile-Leu-Asn-NH<sub>2</sub>

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Under such circumstances, it is desired to provide a VIP derivative having VIP activities higher than those of VIP itself. Specifically, if such a VIP derivative has doubled activities, payability can be sufficiently secured even when it is prepared by the liquid-phase or solid phase process. In some cases, a possibility of inexpensive preparation of such a VIP derivative by gene recombination can be expected. In view of this, in the present invention, the problem to be solved by the invention is to provide a newly designed VIP derivative. VIP has a precursor of its own, the structure of which is revealed in the below-mentioned article (d). It is known that -Asn-Gly-Lys-Arg- in this structure undergoes several steps of enzymatic reactions to finally provide a C-terminal amide group, namely Asn-NH<sub>2</sub>, of VIP. However, no intermediate products in the intermediate steps are known. Thus, there are no substances conceived as VIP derivatives. When the substance having an amide group at the C-terminal is converted into a substance having a carboxyl group at the C-terminal, the activities are generally notably lowered. However, what happens in the case of a VIP derivative is not elucidated. Under such circumstances, the inventors of the present invention have attempted to design a novel substance with a view to finding a

VIP derivative having activities higher than those of VIP itself.

(d) N. Itoh, H. Okamoto et al.; Nature 304, 547 (1983).

5 As a result of investigations, usefulness was found in several newly designed substances. Certain designed substances have activities higher than those of natural VIP even though it has a carboxyl group at the C terminal. It was found that all the substances  
10 had a higher activity of relaxing the smooth muscles of a bronchus and/or a higher hypotensive activity than those of natural VIP.

The present invention has been completed based on such findings.

15 The present invention will now be described in detail.

The substance of the present invention is a novel peptide represented by a primary structural formula:

20 His-Ser-Asp-Ala-Val-Phe-Thr-Asp-  
Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
X-Ala-Val-Lys-Lys-Tyr-Leu-Asn-  
Ser-Ile-Leu-Asn-Gly-Y  
25 (wherein X is Met or Leu; and Y is OH, Lys-OH,  
Arg-OH, Lys-Arg-OH, or Lys-NH<sub>2</sub> when X is Met, or Y is  
Lys-NH<sub>2</sub>, Lys-OH, or Lys-Arg-OH when X is Leu). Here,  
-NH<sub>2</sub> stands for an amide group at the C-terminal,  
while -OH stands for a carboxyl group at the C-terminal.

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The invention provides a process for synthesizing a peptide having the above defined, primary structure, which comprises the steps of attaching a C-terminal amino acid having been protected with a tert-butoxycarbonyl group at the N-terminal to a carrier through an amide group or an ester group, eliminating the tert-butoxycarbonyl group, condensing a second amino acid having a protective group on the N-terminal with the resulting C-terminal amino acid, then effecting the condensations one after another in sequence of the structure of the intended peptide with the subsequent amino acids, eliminating the protective groups from the peptide and taking the obtained peptide out of the carrier.

The process may be followed by purifying the obtained peptide by the ion exchanging chromatography. It is preferred that the starting amino acid has a protective group on a reactive group of the side chain thereof.

Various novel designs in the present invention were made for the following reasons. Since VIP is produced from the known precursor through several steps of enzymatic reactions, a substance wherein X

is Met and Y is OH, Lys-OH, or Lys-Arg-OH was designed as one conceived to be produced in the intermediate step. A substance wherein X is Met and Y is Arg-OH was designed as one having Arg directly bonded to Gly, which Arg is a basic amino acid like Lys, and a substance wherein X is Met and Y is Lys-NH<sub>2</sub> was designed as one having the C-terminal of a basic amino acid converted into an amide group to further increase the basicity, though they do not occur in nature.

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Since Met at the 17th position of VIP is subject to oxidation so that inactivation due to oxidation is apprehended, substitution of Met in the 17th position with Leu which is resistant to oxidation was designed. Although such a sequence is not found in the natural substance, a substance wherein X is Leu and Y is Lys-OH, Lys-Arg-OH, or Lys-NH<sub>2</sub> was designed also in this case with consideration being given to the route of several steps of enzymatic reactions from the above-mentioned known precursor.

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The substance of the present invention is confirmed by a peptide map after enzymatic decomposition and amino acid analysis values after hydrolysis. For further assuring identification,  $[\alpha]_D$  and the retention time in HPLC as physical constants are shown

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in Table 1. The apparatus and conditions for measuring  $[\alpha]_D$  are as follows:

apparatus: JASCO DIP-140 digital polarimeter  
manufactured by Nippon Bunko Co.

5 conditions: Na lamp 589 nm, temperature: 22°C  
cell: 100 nm

integration time: 5 sec

concentration: 0.2 % (in 0.1 N acetic acid)

The column, solvent, detection, etc. in HPLC are as follows:

10 column: YMC ODS 5  $\mu$   $\phi$ 4.6 mm X L 250 mm

solvent: 0.1 % TFA-26.4 %  $\text{CH}_3\text{CN}$ -73.5 %  $\text{H}_2\text{O}$

detection: 215 nm

conditions: 1.0 ml/min, isocratic/elution

15 Table 1

X	Y	$[\alpha]_D^{22}$	retention time (min)
Met	OH	-61.2	13.5
	Lys-OH	-53.9	10.2
	Arg-OH	-53.3	10.9
	Lys-Arg-OH	-60.0	7.5
	Lys-NH <sub>2</sub>	-52.9	8.8
Leu	Lys-NH <sub>2</sub>	-52.8	11.1
	Lys-OH	-56.3	12.4
	Lys-Arg-OH	-54.9	8.9

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The substance of the present invention can be synthesized by the known solid-phase or liquid-phase process. For example, it may be synthesized using a peptide Synthesizer Model 990B manufactured by Beckman by the solid phase process generally called the "Merrifield process."

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A C-terminal amino acid involved in the substance of the present invention, which is protected with a tert-butoxycarbonyl group (hereinafter referred to briefly as "Boc") at the N-terminal, attached to a styrene resin is support through an amide or ester bond. Specifically, Boc-Gly, Boc-Lys (Cl-Z), or Boc-Arg (Tos) is bonded to, for example, a benzhydrylamine resin, a p-methylbenzhydrylamine resin, or a chloromethyl resin. Subsequently, Boc is eliminated with an acid. An amino acid second from the C-terminal which is preliminarily protected at the N-terminal and, if necessary, at a functional group in the side chain is condensed with the above-mentioned amino acid to form a peptide bond. In this case, the protected amino acid is used in an amount of 3 to 5 times the theoretical amount. Dicyclohexylcarbodiimide (hereinafter referred to briefly as "DCC") or a mixture of DCC and 1-hydroxybenzotriazole (hereinafter referred to briefly as "HOBr") is used as the condensing

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agent. The completion of the reaction is confirmed by a point where the reaction of an amino group with ninhydrin becomes negative. In this manner, amino acids protected at the N-terminal and, if necessary, at a functional group in the side chain are sequentially condensed in accordance with the amino acid sequence in the primary structural formula of the substance of the present invention to finally obtain the substance of the present invention having both the functional group and the N-terminal protected. Finally, a treatment with hydrogen fluoride is conducted to eliminate the protective group and the resin from the substance of the present invention. In this treatment, anisole and dimethyl sulfide are added in order to prevent the side reaction. A crude product obtained by removing hydrogen fluoride may be purified by ion exchange chromatography using CM-cellulose or the like. The purity is confirmed by high performance liquid chromatography. If necessary, further purification may be effected by preparative high-performance liquid chromatography to obtain the substance of the present invention in purified form. Confirmation of the purity and the structure may be made by high-performance liquid chromatography, peptide map, amino acid analysis, etc.

The substances according to the present invention, which are VIP derivatives, have a higher activity of relaxing the smooth muscles of a bronchus and/or a higher hypotensive activity than those of VIP itself.

5 Thus, the substances of the present invention have bronchodilative and hypotensive activities, and hence are expected to have usefulness thereof as an asthma treatment drug and a hypotensive drug in future. Since some of the derivatives have a 10 carboxyl group at the C-terminal, they provide a possibility of mass production by gene recombination. Thus, they can be used in place of expensive VIP itself.

Example 1

15 1.25 g of Boc-Lys(Cl-Z)-O-Resin (Boc-Lys(Cl-Z) content: 0.4 m mole/g, manufactured by Peninsula Labs.) was placed in a reaction vessel of a Peptide Synthesizer Model 990B manufactured by Beckman and stirred in  $\text{CH}_2\text{Cl}_2$  for 2 hours to be swollen. 20 Subsequently, Boc-Gly is reacted according to the procedure consisting of the following steps:

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(1) washing three times with 20 ml of  $\text{CH}_2\text{Cl}_2$ ;

(2) preliminarily washing with 20 ml of a  $\text{CH}_2\text{Cl}_2$  solution of 40 % of TFA and 0.05 % of indole;

(3) deblocking with 20 ml of a  $\text{CH}_2\text{Cl}_2$  solution of 40 % of TFA and 0.05 % of indole;

5 (4) washing three times with 20 ml of  $\text{CH}_2\text{Cl}_2$ ;

(5) washing with 20 ml of MeOH;

(6) washing three times with 20 ml of  $\text{CH}_2\text{Cl}_2$ ;

10 (7) preliminarily washing with 20 ml of a  $\text{CH}_2\text{Cl}_2$  solution of 10 % of TEA;

(8) neutralizing with 20 ml of a  $\text{CH}_2\text{Cl}_2$  solution of 10 % of TEA;

(9) washing three times with 20 ml of  $\text{CH}_2\text{Cl}_2$ ;

15 (10) dissolving 2.5 m moles of a Boc-protected amino acid and 2.5 m moles of HOBr in a mixed liquid of 7.5 ml of DMF and 7.5 ml of  $\text{CH}_2\text{Cl}_2$  and adding the resulting solution; and

(11) adding 5 ml of a 0.5M  $\text{CH}_2\text{Cl}_2$  solution of DCC and conducting a reaction for 2 hours.

20 Introduction of Boc-Gly is completed by the above-mentioned procedure.

The steps (1) to (11) are repeated to form a peptide chain starting with sequence Gly toward an N-terminal.

25 The protected amino acids are added in the

following order. Every amino acid was added in an amount of 2.5 m moles which is five times that of Lys bonded to the resin.

5                   Boc-Asn 0.58 g/HOBt 0.34 g (The presence of HOBt improves the condensation yield and prevent racemization. However, when His is present, HOBt is not used because imidazole undergoes a change. In this case, 2.5 m moles of HOBt is added to every amino acid except for His which is a final N-terminal in coupling). Boc-Leu · H<sub>2</sub>O 0.62g, Boc-Ile · 1/2H<sub>2</sub>O 0.60g, BOC-Ser(Bzl) 0.74g, Boc-Asn 0.58g, Boc-Leu · H<sub>2</sub>O 0.62g, Boc-Tyr(Br-Z) 1.24g, Boc-Lys(Cl-Z) 1.00g, Boc-Lys(Cl-Z) 1.00g, Boc-Val 0.54g, Boc-Ala 0.47, Boc-Leu · H<sub>2</sub>O 0.62g, 15                   Boc-Gln 0.62, Boc-Lys(Cl-Z) 1.00g, Aoc-Arg(Tos) · 1/4 EtoAc · 1/2 H<sub>2</sub>O 1.18g, Boc-Leu · H<sub>2</sub>O 0.62g, Aoc-Arg(Tos) · 1/4EtoAc · 1/2 H<sub>2</sub>O 1.18g, Boc-Thr(Bzl) 0.77g, Boc-Tyr (Br-Z) 1.24, Boc-Asn 0.58g, Boc-Asp(OcHex) 0.79g, Boc-Thr(Bzl) 20                   0.77g, Boc-Phe 0.66g, Boc-Val 0.54g, Boc-Ala 0.47g, Boc-Asp(OcHex) 0.79g, Boc-Ser (Bzl) 0.74g, Boc-His(Tos) 1.03g (HOBt is not added in this case) When the above-mentioned procedure is completed, the following protected peptide is synthesized on 25                   the resin.

Boc-His (Tos)-ser (Bzl)-Asp (OcHex)-Ala-  
Val-Phe-Thr (Bzl)-Asp (OcHex)-Asn-Tyr (Br  
-Z)-Thr (Bzl)-Arg (Tos)-Leu-Arg (Tos)-Lys  
(Cl-Z)-Gln-Leu-Ala-Val-Lys (Cl-Z)-Lys  
(Cl-Z)-Tyr (Br-Z)-Leu-Asn-Ser (Bzl)-Ile-  
Leu-Asn-Gly-Lys (Cl-Z)- resin.

5 This protected peptide attached to the resin is subjected to the aforementioned steps (1) to (9), filtered, and dried in a desiccator over night.

10 3.25 g of a dried protected peptide attached to the resin is obtained. 1.3 g thereof is treated with 30 ml of hydrogen fluoride in the presence of 2 ml of anisole and 0.5 ml of DMS at 0°C for one hour. The hydrogen fluoride is distilled off. The residue is washed with a mixed liquid of anhydrous ether and n-hexane (1:1), washed with only anhydrous ether, and sufficiently dried. The peptide is dissolved in 50 ml of 10 % acetic acid, and the undissolved resin is filtered out. The obtained solution is filtered through a 0.22μ Millipore filter, followed by freeze 15 drying. 628 mg of a crude peptide is obtained.

20 Subsequently, the crude peptide is subjected to CM-cellulose column (2.5φ 30h cm), chromalography, and eluted (6.5 ml/fr) with AcONH<sub>4</sub> (pH: 7.0) having 25 a straight line inclination ranging from 0.05 M to

0.5 M to elute the desired peptide in fr. No. 112 to 119. Thus, 104 mg of a partially purified peptide is obtained. It is then subjected to high-performance liquid chromatography under the following conditions:

5 column: YMC ODS 5 $\mu$   $\varnothing$ 10mm X L 250 mm,

solvent: R and S mentioned below are prepared, a solvent having a straight line inclination ranging from a mixed liquid of 65 % of R and 35 % of S to a mixed liquid of 40 % of R and 60 % of S is used:

R:  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$

(1:99, containing 0.1 % of TFA)

S:  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$

(6:4, containing 0.1 % TFA),

15 rate of flow: 3 ml/min

10.0 mg of a pure peptide is obtained from 17.4 mg of the partially purified peptide by said chromatography. In this case, the yield is 8.5 % when calculated from coupling with Asn. The peptide map and amino acid analysis values are examined to confirm that the product is a substance according to the present invention. Further, the  $[\alpha]_D^{22}$  and retention time are examined as values of physical properties. In this Example, the substance according

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to the present invention wherein X is Leu and Y is Lys-OH is obtained. The abbreviations used in the description are according to the nomenclature of IUPAC-IUB Commission [Eur. J. Biochem. 138, 9 (1984)], and are as follows.

5 Bzl: Benzyl

Cl-Z: 2-Chlorobenzylloxycarbonyl

Tos: 4-Toluenesulfonyl

Br-Z: 2-Bromobenzylloxycarbonyl

10 OcHex: Cyclohexyl ester

The results of amino acid analysis, conducted by the inventors of the present invention, of the substance obtained in this example are shown below for reference.

15 The hydrolysis reaction is conducted in 6N HCl (containing 0.1 % of phenol) at 110°C for 20 hours, followed by analysis with a Hitachi amino acid analyzer Model 835. As described below, observed values well agrees with theoretical values, thus substantiating that the compound is a desired one according to the present invention.

<u>Amino acid</u>	<u>Theoretical value</u>	<u>Observed value</u>
Asp	5	5.00
Thr	2	1.90

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	Ser	2	1.79
	Glu	1	1.04
	Gly	1	1.04
	Ala	2	2.00
5	Val	2	2.00
	Ile	1	0.95
	Leu	4	4.00
	Tyr	2	1.98
	Phe	1	1.00
10	Lys	4	3.95
	His	1	1.06
	Arg	2	1.99

## Example 2

Substantially the same procedure as in Example 1  
 15 is repeated until completion of sequential  
 introduction of protected amino acids except that  
 1.25 g of Boc-Met is used instead of 0.62 g of  
 Boc-Leu·H<sub>2</sub>O introduction of the 17th amino acid from  
 the N-terminal.

20 When the procedure is fully completed, the  
 following protected peptide is synthesized on the  
 resin.

Boc-His (Tos)-Ser (Bzl)-Asp (OcHex)-Ala-  
 Val-Phe-Thr (Bzl)-AsP (OcHex)-Asn-Tyr (Br  
 -z)-Thr (Bzl)-Arg (Tos)-Leu-Arg (Tos)-Lys

(Cl-Z)-Gln-Met-Ala-Val-Lys(Cl-Z)-Lys

(Cl-Z)-Tyr(Br-Z)-Leu-Asn-Ser(Bzl)-Ile-  
Leu-Asn-Gly-Lys(Cl-Z)- resin.

After the aforementioned steps (1) to (9) are

5 performed, the protected peptide attached to the resin is filtered and dried in a desiccator over night. 3.4 g of the dried protected peptide is obtained.

10 1.7 g of the dried protected peptide is treated with 30 ml of hydrogen fluoride in the presence of 2 ml of anisole and 0.5 ml of DMS at 0°C for one hour. The hydrogen fluoride is distilled off, and the residue is washed with a mixed liquid of anhydrous ether and n-hexane (1:1), washed with only anhydrous ether, and sufficiently dried. The peptide is dissolved in 50 ml of 10 % acetic acid, and the undissolved resin is filtered out. The obtained solution is filtered through a 0.22  $\mu$  Millipore filter, followed by freeze drying. 834 mg of a crude peptide is obtained. Subsequently, the crude peptide is

15 subjected to CM-cellulose column (2.5 $\phi$  x 30h cm) chromatography, and eluted (6.5 ml/fr) with AcONH<sub>4</sub> (pH: 7.0) having a straight line inclination ranging from 0.05 M to 0.5 M to elute the desired peptide in fr. No. 109 to 121. Thus, 303 mg of a partially

20 purified peptide is obtained. It is then subjected to

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high-performance liquid chromatography under the following conditions:

column: YMC ODS 5  $\mu$   $\phi$  10 mm X L 250 mm,

solvent: R and S mentioned below are prepared,

5 a solvent having a straight line inclination ranging from a mixed liquid of 65 % of R and 35 % of S to a mixed liquid of 40 % of R and 60 % of S is used:

R:  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$

10 (1:99, containing 0.1 % of TFA)

S:  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$

(6:4, containing 0.1 % of TFA),

rate of flow: 3 ml/min.

11.5 mg of a pure peptide is obtained from 16.3 mg .. of the partially purified peptide by said chromatography.

15 In this case the yield is 24.3 % when calculated from coupling with Asn. The peptide map and amino acid analysis values are examined to confirm that the product is a substance according to the present

20 invention. Further, the  $[\alpha]_D$  and retention time are examined as values of physical properties. In this Example, the substance according to the present invention wherein X is Met and Y is Lys-OH is obtained.

25 The abbreviations in the description are the same as in Example 1.

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The results of amino acid analysis, conducted by the inventors of the present invention, of the substance obtained in this Example are shown below for reference.

The hydrolysis reaction is conducted in 6N HCl (containing 0.1 % of phenol) at 110°C for 20 hours, followed by analysis with a Hitachi amino acid analyzer Model 835. As described below, observed values well agrees with theoretical values, thus substantiating that the compound is a desired one according to the present invention.

	<u>Amino acid</u>	<u>Theoretical value</u>	<u>Observed value</u>
	Asp	5	5.00
5	Thr	2	1.94
	Ser	2	1.80
10	Glu	1	1.06
	Gly	1	1.06
15	Ala	2	2.05
	Val	2	2.00
20	Ile	1	1.00
	Leu	3	3.07
	Tyr	2	2.02
25	Phe	1	1.01
	Lys	4	3.98
	His	1	1.07
	Arg	2	2.02
	Met	1	1.03

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The effects of the present invention will now be shown by the following Experimental Example.

**Experimental Example**

**Samples:**

5        The substance of the present invention is used as a specimen. A natural VIP (hereinafter referred to briefly as "VIP-NH<sub>2</sub>") and a synthetic VIP derivative (hereinafter referred to briefly as "VIP-OH") in which the C-terminal amide group of VIP has been  
10        substituted with a carboxyl OH group are prepared and used as control samples.

**Methods:**

The following two determinations, A and B, are conducted.

15        A. Determination on Relaxation of Muscle of Bronchus  
              Removed from Guinea Pig:

The juglar vein of a male guinea pig weighing 300 g or more is cut, causing the guinea pig to be bled to death. The guinea pig undergoes thoracotomy.  
20        Immediately after the thoracotomy, 3 cm of the trachea is taken out. The trachea is immediately put in a Krébs solution. It is then cut into seven equal rings, and the cartilages are connected to one another with a cotton yarn to form a chain. Subsequently, the smooth

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muscle parts and cartilages opposite them are cut, so  
that the smooth muscle parts can be connected to one  
another. The preparation thus prepared is placed in  
an isothermic glass cell having an internal capacity  
of about 7 ml and suspended from above. The lower  
part of the preparation is fixed, while the upper  
part is connected to an isometric transducer, to  
which 0.5 g of a load has been applied, in order to  
determine the relaxation reaction. Sufficient amounts  
of 95 % oxygen and 5 % carbon dioxide gas are blown  
into the preparation. Further, a Krebs solution of  
37°C containing  $6.5 \times 10^{-6}$  M histamine is dropped on  
the preparation from above at a flow rate of 3.3 ml/min.  
The relaxation reaction takes place to an extent  
proportional to the amount of a sample added to the  
dropped solution. By utilizing this relationship, the  
relaxation activity against bronchial smooth muscle  
with respect to the substance of the present invention  
is compared with that of VIP-NH<sub>2</sub>. VIP-NH<sub>2</sub> is used at  
a concentration of  $10^{-5}$  M and dropped in amounts of 40  
μl and 80 μl. The substance of the present invention  
is also used at a concentration of  $10^{-5}$  M and dropped  
in amounts of 10 μl and 20 μl. The value of the area  
(height x time) in relaxation of each case is determined.  
The specific activities are obtained by taking the area

value of VIP-NH<sub>2</sub> as 100 % and determining the relative ratio of the area value of each case. Thus, the relaxation reactions are compared by the four-point assay method, with respect to the above-mentioned 5 determination method, reference may be made to the following articles (e) and (f):

(e) W.L.M. Perry, Pharmacological Experiments on Isolated Preparations: E & S Livingstone LTD. 1968; and

10 (f) Castillo, De Beer, J. Phamac. Exp. Ther. 90, 104 (1947).

B. Determination of Carotid Artery Pressure on Rat:

An SD male rat having a body weight of 200 g is anesthetized by intraperitoneally administering 15 48 % urethane at a dosage of 2.7 ml/kg. In order to inject the rat, cannulation is made to the carotid artery. The cannulation of a transducer for determining the blood pressure is also made to the carotid artery. A sample of 12.5 pmol/kg to 400 pmol/kg 20 is administered into the carotid artery, and the change in blood pressure is plotted with a polygraph to obtain a dose-response curve. The amount of the sample required to produce a drop of 15 mmHg in blood pressure 25 is determined from the curve. The required amount of VIP-NH<sub>2</sub> is taken as 100 %, and the specific activity

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of each sample is obtained by calculating the relative ratios.

With respect to the above-mentioned determination method, reference may be made to the following article  
5 (g):

(g) Suzuki, Araki, and Tachibana, Yakugaku Zasshi, 99 (2), 172 (1979).

Results:

The results are shown in Table 2. In the Table, the value in col. A is the specific activity (%) obtained by the above-mentioned method A, while the value in col. B is the specific activity (%) obtained in the above-mentioned method B. As can be seen from Table 2, the substance of the present invention has a higher activity of relaxing the smooth muscle of a bronchus and/or and a higher hypotensive activity than those of the natural VIP.

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Table 2

Sample	X	Y	A	B
Speci- men	Met	OH	70	110
		Lys-OH	300	200
		Arg-OH	220	250
		Lys-Arg-OH	460	180
		Lys-NH <sub>2</sub>	140	80
	Leu	Lys-NH <sub>2</sub>	300	100
		Lys-OH	410	220
		Lys-Arg-OH	410	80
	Control		100	100
	VIP - NH <sub>2</sub>		60	70
VIP - OH				

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Claims:

1. A peptide having the primary structural formula:

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-  
Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
X-Ala-Val-Lys-Lys-Tyr-Leu-Asn-  
Ser-Ile-Leu-Asn-Gly-Y

in which X is Met and Y is OH, Lys-OH, Arg-OH, Lys-  
Arg-OH or Lys-NH<sub>2</sub> or X is Leu and Y is Lys-NH<sub>2</sub>, Lys-  
OH or Lys-Arg-OH.

2. A peptide as claimed in Claim 1, in which X is  
Met and Y is OH, Lys-OH, Arg-OH, Lys-Arg-OH or Lys-  
NH<sub>2</sub>.

3. A peptide as claimed in Claim 1, in which X is  
Leu and Y is Lys-NH<sub>2</sub>, Lys-OH or Lys-Arg-OH.

4. A process for synthesizing a peptide having the primary structural formula as defined in Claim 1, which comprises the steps of attaching a C-terminal amino acid having been protected with a tert-butoxycarbonyl group at the N-terminal to a carrier through an amide group or an ester group, eliminating the tert-butoxycarbonyl group, condensing a second amino acid having a protective group on the N-terminal with the resulting C-terminal amino acid, then effecting the condensations one after another in sequence of the structure of the intended peptide with the subsequent amino acids, eliminating the protective groups from the peptide and taking the obtained peptide out of the carrier.

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5. A process as claimed in Claim 4, which further comprises purifying the obtained peptide by the ion exchanging chromatography.

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6. A process as claimed in Claim 4, in which the starting amino acid has a protective group on a reactive group of the side chain thereof.

5 7. Pharmaceutical composition comprising a compound according to any of claims 1 to 3 and a pharmaceutically acceptable carrier.

10 8. Use of the compounds of any of claims 1 to 3 in the preparation of a medicament having bronchodilative and hypotensive activity.

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Claims for Austria, Spain and Greece

1. Process for synthesizing a peptide having the primary structural formula:

5 His-Ser-Asp-Ala-Val-Phe-Thr-Asp-  
Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
X-Ala-Val-Lys-Lys-Tyr-Leu-Asn-  
Ser-Ile-Leu-Asn-Gly-Y

10 in which X is Met and Y is OH, Lys-OH, Arg-OH, Lys-  
Arg-OH or Lys-NH<sub>2</sub> or X is Leu and Y is Lys-NH<sub>2</sub>, Lys-OH  
or Lys-Arg-OH which comprises the steps of attaching a  
15 C-terminal amino acid having been protected with a  
tert-butoxycarbonyl group at the N-terminal to a car-  
rier through an amide group or an ester group, elim-  
inating the tert-butoxycarbonyl group, condensing a  
second amino acid having a protective group on the  
N-terminal with the resulting C-terminal amino acid,  
then effecting the condensations one after another in  
sequence of the structure of the intended peptide with  
20 the subsequent amino acids, eliminating the protective  
groups from the peptide and taking the obtained pep-  
tide out of the carrier.

25 2. A process as claimed in Claim 1, which further  
comprises purifying the obtained peptide by the ion  
exchanging chromatography.

30 3. A process as claimed in Claim 1, in which the  
starting amino acid has a protective group on a re-  
active group of the side chain thereof.

4. Use of the final products synthesized in any of  
Claims 1 to 3 in the preparation of a medicament hav-  
ing bronchodilative or hypotensive activity.





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⑵ Peptide, process for synthesizing it, pharmaceutical composition containing it and use.

⑶ A peptide having the following structure:

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-  
Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-  
X-Ala-Val-Lys-Lys-Tyr-Leu-Asn-  
Ser-Ile-Leu-Asn-Gly-Y

(wherein X is Met or Leu; and Y is OH, Lys-OH, Arg-OH, Lys-Arg-OH, or Lys-NH<sub>2</sub> when X is Met, or Y is Lys-NH<sub>2</sub>, Lys-OH, or Lys-Arg-OH when X is Leu), a process for synthesizing it, a pharmaceutical composition containing it, and its use in the preparation of a medicament having bronchodilative and hypotensive activity are disclosed.

EP 0 241 926 A3



European Patent  
Office

EUROPEAN SEARCH REPORT

0241926

Application Number

EP 87 10 5570

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	US-A-3 862 927 (SAID) * Whole document * ---	1,4,7	C 07 K 7/10 C 07 K 1/04 A 61 K 37/02
A	US-A-3 880 826 (SAID) * Whole document * ---	1,4,7	
P, A	US-A-4 605 641 (BOLIN) * Whole document * ---	1,4,7	
A	CHEMICAL ABSTRACTS, vol. 81, 1974, page 170, abstract no. 223999m, Columbus, Ohio, US; V. MUTT et al.: "Structure of the porcine vasoactive intestinal octacosapeptide. Amino acid sequence. Use of kallilrein in its determination", & EUR. J. BIOCHEM. 1974, 42(2), 581-9 * Abstract * ---	1,4,7	
A	CHEMICAL ABSTRACTS, vol. 100, 1984, page 117, abstract no. 80580k, Columbus, Ohio, US; S.R. BLOOM et al.: "Diarrhea in Vipoma patients associated with cosecretion of a second active peptide (peptide histidine isoleucine) explained by single coding gene", & LANCET 1983, 2(8360), 1163-5 * Abstract * ---	1,4,7	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 07 K 7/00 A 61 K 37/00
A	CHEMICAL ABSTRACTS, vol. 103, 1985, page 208, abstract no. 173266q, Columbus, Ohio, US; T. TSUKADA et al.: "Structure of the human vasoactive intestinal polypeptide gene", & DNA 1985, 4(4), 293-300 * Abstract * -----	1,4,7	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	09-12-1988	RAJIC M.	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
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